EXPERIMENTAL MUTABILITY OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS. PART I. PROPERTIES OF MUTANTS INDUCED BY ALKYLATING COMPOUNDS

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Summary

The induced mutability of the Venezuelan equine encephalomyelitis virus affecting many of its characters was studied. Alkylating compound (formaldehyde, nitrosomethylurea and ethyleneimine) were used as mutagens.

It was established that nitrosomethylurea posessed the greattest mutagenic activity, the frequency of mutations induced being 42,5%. There was no difference in this respect between formaldehyde and ethyleneimine (both 33,0%).

In the spectrum of mutations affecting the pathogenicity formaldehyde surpasses two other mutagens. Besides it induced mutations characterized by the formation of small plaques.

The changes of pathogenicity caused by mutations were associated with the changes of certain other genetic characters.

EXPERIMENTAL MUTABILITY OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Part I: Properties of Mutants Induced by Alkylating Compounds

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Introduction

In recent years a number of studies have been devoted to the directed mutability of human and animal viruses when their genetic apparatus is acted upon by certain chemical compounds [1-6].

The purpose of the present work is to study the mutability of certain properties of Venezuelan equine encephalitis virus under the influence of alkylating compounds.

Materials and Methods

The experiments were carried out with the "standard" strain of Venezuelan equine encephalitis (VEE) virus, obtained from the live culture collection of the D. I. Ivanovskiy Institute of Virology of the USSR Academy of Medical Sciences. Chick embryo cell (CEC) cultures were prepared by the ordinary method of trypsinization and grown on a culture medium consisting of 0.5% hydrolizate of lactalbumin in Hanks' solution and 10% normal bovine serum.

Negative colonies were prepared by the method of Dulbecco and Vogt [7] as modified by Andzhaparidze and Stepanova [8]. Plaques 5-6 mm in diameter were considered large (S^+), and those 1 mm in diameter or less were considered small (S^-).

Alkylating compounds were applied to the intracellular virus during replication. The following concentrations of mutagens were used: formaldehyde (F) -- $7.5 \cdot 10^{-4}$ and $3.75 \cdot 10^{-4}$ M; N-nitrosomethyl-urea

10

(N-NMU) -- 1:1000 and 1:2000; ethylenimine (EI) -- 1:1000. Solutions of all mutagens were prepared on a 0.5% solution of hydrolizate of lactalbumin containing 2% normal bovine serum.

After formation of a monolayer, a viral suspension, prepared from the brain of white mice inoculated with Venezuelan equine encepahlitis virus, was introduced into the tissue cultures at the rate of 5-10 plaque-forming particles (PFP) per cell. After the virus was adsorbed for 1.5 hours at 37°C, the cells were washed twice, after which a culture medium (an 0.5 solution of hydorizate of lactalbumin in Hanks' solution with 2% normal bovine serum) containing various concentrations of mutagens was added and placed in a thermostat at 37°C. Inoculated tissue cultures to which ordinary culture medium was added, instead of mutagens, served as the control. After the intracellular virus was exposed to formaldehyde for 5 and 18 hours and to N-NMU and EI for 3 hours, the culture medium was removed, and the cells were washed three times with Hanks' solution, removed from the surface of the glass mechanically, resuspended in Hanks' solution, and disrupted by freezing and thawing. The mutant clones were isolated by the negative colony method.

Nonvarietal white mice weighing 7-8 grams, guinea pigs weighing 250-300 grams and Chinchilla rabbits weighing 2.0-2.5 kg were used to determine the pathogenicity of the mutants. The mice were inoculated intracerebrally with 0.03 ml of virus-containing material and with 0.3 ml subcutaneously, and the guinea pigs and rabbits subcutaneously with 0.3 and 0.5 ml respectively. Clones which did not produce disease when inoculated subcutaneously were considered peripherally apathogenic (sc^-). If the virus titer (log LD_{50}) in this method of inoculation was 2.0 or less, the clone was considered to possess reduced peripheral activity ($sc^\pm$). Clones with a virus titer greater than 2.5 were considered pathogenic (sc^+). Clones having a titer of 4.0 log EID_{50} and greater when inoculated intracerebrally were considered pathogenic (ic^+).

To study thermostability of the mutants, the virus-containing liquids were heated for 20 minutes in glass test tubes in a water bath having an automatically regulated temperature of 55°C. If the titer (log EID_{50}) decreased by 4 or more, the mutant was considered thermolabile (T_{55}^-); if the titer decreased by less than 3.0, the mutant was considered thermostable (T_{55}^+); if the decrease in titer was between 3.0 and 4.0, the mutant was considered intermediate ($T_{55}^\pm$).

The clones which produced viremia in the infected animals were studied in experiments on 250-300 g guinea pigs. The animals were inoculated subcutaneously with 0.2 ml of undiluted virus-containing culture liquid. Blood was taken through cardiac puncture after periods of 2, 3, 4 and 5 days and titrated in a CEC culture.

The method described by Podgodina and her co-workers [9] was used

to prepare organ extracts of the white mice. The presence of virus was determined by its cytopathic effect in a CEC culture.

The antigenic properties of the mutants were tested on 2.0-2.5 kg Chinchilla rabbits. After background blood samples were taken, the animals were immunized twice at an interval of 21 days by subcutaneous injections of 0.5 and 1 ml of virus-containing culture liquid respectively. Blood was drawn from the marginal vein of the ear every 7 days for 6 weeks. The growth dynamics of virus-neutralizing antibodies were studied through the neutralization reaction in a CEC culture, and that of hemagglutination-inhibiting antibodies through the hemagglutination-inhibition (HI) test.

The immunogenic properties of the virus clones were tested on white mice. Animals weighing 7-8 g received a single subcutaneous dose of 0.3 ml of undiluted virus-containing liquid (experiment) or of Hanks' solution (control). Twelve days after injection the experimental and control groups were inoculated with exponentailly increasing solutions (10^{-1}-10^{-8}) of the pathogenic strain of VEE. Observations were made for 12 days, then the resistance index was computed by the method of Reed and Muench [10].

Experimental Results

It was found that a population of intact VEE virus is heterogenous and consists of particles which under agar form both large (5-6 mm) and small (0.8-1 mm) round transparent plaques with distinct even edges. The number of small plaques did not exceed 11.9% (table 1).

When formaldehyde acted on the virus, the make-up of the virus population changed in favor of forming small-plaque variants, and their yield increased as the exposure time of virus and mutagen was extended. It must be noted that under the influence of formaldehyde very small plaques (0.3-0.5 mm) appeared, which were not found in the untreated control virus. When the virus was treated with N-NMU and EI, mutations in favor of forming small plaques were not noted. The plaque-size index in formaldehyde-induced mutants remained stable even after 75 passages in a CEC tissue culture.

When clones produced by the action of formaldehyde were titrated intracerebrally and subcutaneously on white mice (table 2), mutants apathogenic in peripheral inoculation (sc^-) and mutants with reduced peripheral activity ($sc^\pm$) were both noted. Mutants with changed pathogenicity in intracerebral inoculation were not observed. Nor did mutants appear which were entirely apathogenic for mice. All mutants with lost or reduced peripheral pathogenicity for mice were produced from small plaques.

Table 1

The Effect of Alkylating Compounds on Plaque Size in Venezuelan Equine Encephalitis Virus

Mutagen	Concentration of mutagen, exposure	Total plaques	Small plaques: number	Small plaques: %
F	$7.5 \cdot 10^{-4}$ M, 5 hrs;	83	58	69.8
	$3.75 \cdot 10^{-4}$ M, 18 hrs	125	106	84.9
N-NMU	1:1000, 3 hrs	167	17	10.2
	1:2000, 3 hrs	135	15	11.1
EI	1:1000, 3 hrs	201	21	10.45
Control virus	-	159	19	11.9

Table 2

The Effect of Alkylating Compounds on the Pathogenicity for Mice of Venezuelan Equine Encephalitis Virus

Character of mutations in pathogenicity	Mutagen and S-index of clones: F		N-NMU		EI		Control virus	
	S^+	S^-	S^+	S^-	S^+	S^-	S^+	S^-
ic^+sc^+	20	55	5	7	8	4	17	33
$ic^+sc^\pm$	0	23	0	9	0	6	0	0
ic^+sc^-	0	15	0	0	0	0	0	0
All clones tested	20	93	5	16	8	10	17	33
Clones with changed peripheral activity (%)	33.6		42.5		33.3		0	

When clones derived from treatment of the virus with N-NMU were titrated on white mice, some of them showed reduced peripheral activity. Other mutations with respect to pathogenicity did not appear. All the mutants produced were characterized by small plaques.

Among the clones tested after treatment of the virus with EI, mutants with reduced peripheral activity were also found, and all of them also were characterized by small plaques.

In contrast to the experimental clones, both small and large-plaque clones produced from the control virus were identically virulent.

To conduct a longer study of certain indices (pathogenicity for guinea pigs and rabbits, viremia, virus distribution in the bodies of white mice, thermoresistance, antigenic and immunogenic properties) we selected 5 mutants induced by formaldehyde (F), 4 by N-NMU and 4 by EI.

One of the formaldehyde mutants (F_2) was apathogenic when inoculated subcutaneously in white mice, while the rest showed reduced peripheral activity. In terms of plaque size, they all had the S^- character.

In studying the peripheral pathogenicity of selected mutants for guinea pigs and rabbits, it was shown (table 3) that only 3 formaldehyde mutants, 2 N-NMU mutants and one EI mutant retained their pathogenicity for these animals, while all other mutants were completely apathogenic for the experimental animals.

Table 3

Properties of Mutants of Venezuelan Equine Encephalitis Virus with Respect to Viremia, Thermoresistance and Pathogenicity for Animals

	Intense viremia					Moderate viremia			Low or absent viremia				
Animals	F 1	N-NMU 17	N-NMU 20	EI 5	EI 15	F 3	EI 10	N-NMU 12	F 2	N-NMU 5	EI 2	F 7	F 11
	Pathogenicity (sc)												
White mice	±	±	±	±	±	±	±	±	-	±	±	±	±
Guinea pigs	+	+	+	+	+	+	±	±	-	-	-	-	-
Rabbits	+	+	+	+	+	+	±	±	-	-	-	-	-
	Thermoresistance at 55°C												
	-	±	±	±	±	-	+	+	-	-	-	-	-

Note. The following abbreviations are used in this and the next table: F -- formaldehyde-induced mutants; N-NMU -- nitrosomethyl-urea-induced mutants; EI -- ethylenimine-induced mutants; the numerals indicate the serial numbers of the mutants.

According to the intensity of the viremia induced in animals, all the mutants studied can be divided into three groups: 1) those causing intense viremia -- > 10^4 EID_{50}/ml, 2) those causing moderate viremia -- 10^3 -10^4 EID_{50}/ml, and 3) those causing low viremia -- < 10^3 EID_{50}/ml. As seen from the data in table 3, the level of viremia produced by a given clone in guinea pigs is directly proportional to the degree of that clone's pathogenicity for the given animal.

The clones were also heterogeneous in terms of the T_{55}-character. All formaldehyde-induced mutants proved thermolabile, as well as two mutants, apathogenic for guinea pigs and rabbits, induced by N-NMU and EI. The remaining mutants were characterized as T_{55}^-. Clones derived from the control virus population were thermostable.

Study of the distribution and the accumulation dynamics of the

virus in the organs of white mice showed (table 4) that all the mutants tested were capable of reproducing in the brain and spleen, but the titer level and persistence in these organs depended on the degree of the mutants' pathogenicity.

Table 4

The Dynamics of Virus Accumulation in the Organs of Mice Infected with Induced Virus Clones

Organ	Time after inoculation (in hours)	Virus titer (log EID_{50}/ml)												
		F 1	F 2	F 3	F 7	F 11	N-NMU 5	N-NMU 12	N-NMU 17	N-NMU 20	EI 2	EI 5	EI 10	EI 15
Brain	24	-	-	3.0	-	-	-	-	2.0	2.5	-	-	-	-
	48	2.75	2.0	2.5	2.25	1.75	2.0	4.0	2.75	4.0	2.25	3.5	2.75	2.5
	72	4.0	-	-	-	-	-	-	-	3.0	2.0	4.0	-	-
	96	1.0	-	-	-	-	-	-	-	-	-	2.75	-	-
Liver	24	1.0	-	2.0	-	-	-	1.5	1.75	1.5	-	-	-	-
	48	2.25	-	-	-	-	-	2.25	3.0	3.25	2.0	3.25	1.75	2.25
	72	-	-	-	-	-	-	1.0	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	24	-	-	-	3.0	-	1.0	1.75	2.5	2.0	-	-	-	-
	48	2.0	2.0	3.0	2.25	2.25	1.75	2.5	3.25	2.75	2.2	4.0	2.2	2.0
	72	1.0	-	2.75	-	-	2.25	4.0	2.0	3.0	2.0	4.0	2.25	1.5
	96	-	-	1.0	-	-	-	2.25	-	-	-	2.5	-	-

Virus clones apathogenic for guinea pigs and rabbits were not found in the liver, with the exception of one ethylenimine-induced virus, which was observed in that organ for only 48 hours after inoculation in titer (log EID_{50}/ml); two clones pathogenic for these animals multiplied in the liver and accumulated in quite high titers: 2.25-3.25.

When rabbits were immunized with formaldehyde-induced mutants, quite intensive accumulation of virus-neutralizing antibodies took place (table 5). By the 21st day after the second injection the titer of these antibodies equalled 1:3125 for all the clones studied. By this same time the titer of antihemagglutins reached 1:3413-1:5120. Mutants produced by the action of N-NMU and EI on VEE virus had very low indices of antigenicity. When rabbits were immunized with these mutants, the titer of virus-neutralizing antibodies was 1:25 by the 21st day after the first immunization.

Table 5

Antigenic and Immunogenic Activity of Mutants Induced by Alkylating Compounds

Antibody titer and resistance index	Type of reaction	Variant tested: F 2	F 7	F 11	N-NMU 5	N-NMU 12	EI 2	EI 15	EI 10
21st day after first immunization	NR	1:640	1:640	1:640	1:25	1:25	1:25	1:25	1:25
	HI	1:1066	1:1280	1:1280	1:33	1:53	1:26	1:33	1:40
21st day after second immunization	NR	1:315	1:3125	1:3125	Not immunized				
	HI	1:3413	1:4266	1:5120	Not immunized				
Resistance index		5,5	5,6	5,6	1,0	1,0	2,0	2,0	0

Note. The following abbreviations are used: NR -- neutralization reaction; HI -- hemagglutination-inhibition test.

and the titer of antihemmaglutins was 1:33-1:53 in the same period. A single immunization of mice with formaldehyde mutants caused them to develop resistance to later infection by a pathogenic strain of VEE virus. The resistance index attained a value of 5,5-5,6. Weak immunogenic properties were evident in N-NMU and EI-induced mutants. Their resistance index was no higher than 1,0-2,0.

Discussion

This research showed it was possible, through the action of certain alkylating compounds on VEE virus, to produce mutants exhibiting various changes in pathogenicity for laboratory animals. It was established that N-NMU possessed the highest mutagenic activity with respect to percentage yield of mutants. Ethylenimine and formaldehyde were identical in this regard. However, in terms of the spectrum of mutations in pathogenicity, the most active proved to be formaldehyde, which moreover produced changes in the viral population in favor of forming small-plaque variants. The mutagenic action of formaldehyde on various viruses of transmissable encephalitises was studied by Zasukhina [11]. When formaldehyde acted on the tick-borne encephalitis virus, mutants were produced having reduced peripheral and cerebral activity. After treatment of Western equine encephalitis virus with formaldehyde, the percentage of mutants forming small plaques in a chick fibroblast culture equalled 99, whereas the percentage of such mutants in untreated virus did not exceed 5-8 [12]. According to our data, these same percentages for Venezuelan equine encephalitis virus equalled 84,9 and 11,9 respectively. There are also interesting data in the literature with respect to the action of N-NMU on various viruses. When a virus of the tick-borne encephalitis complex

was treated with this compound [4], small-plaque mutations were formed two times more rarely than apathogenic mutations. The action of N-NMU on the virus of Western equine encephalitis [6] gave rise to mutations with respect to pathogenicity. In experiments with Eastern equine encephalitis [13] mutations in pathogenicity through the action of this compound were not noted. When the virus of Omsk hemorrhagic fever was treated with N-NMU, small-plaque variants were observed [5]. In our experiments N-NMU caused mutations only with respect to pathogenicity. This diverse effect of one and the same chemical compound on various viruses is probably associated with peculiarities in the structure of their nucleic acids.

Changes in virus pathogenicity are known frequently to be associated with changes in certain other genetic characteristics. In our experiments all variants which exhibited reduced pathogenicity for mice were characterized by small plaques. These data correspond to the results also obtained by several other investigators. For example, strains of Western and Eastern equine encephalitis viruses apathogenic for mice were isolated from small plaques [14, 15].

We established a close correlation between pathogenicity and the level of viremia induced by a given clone. Pogodina [16], Gendon and his co-workers [17] and Sarmanova [18] noted a high degree of correlation between viremia and pathogenicity for other viruses. In our experiments mutants with reduced peripheral activity were characterized by preferential reproduction in the spleen of infected animals.

Differing velocities of inactivation are an additional indicator to differentiate between virus variants produced by heating. A close correlation was noted between thermoresistance and the size of negative colonies [19]. Our studies also established a correlation between the thermoresistance and pathogenicity of a given variant.

Some variants, though their peripheral activity was reduced, retained marked antigenicity and immunogenicity, which recommends them for further study as candidates for vaccine strains.

Conclusions

As a result of our studies on the effect of alkylating compounds on VEE virus, it was established that N-NMU possessed the greatest mutagenicity (42.5%), while F and EI were similar in this respect (33.6% and 33.3%).

With respect to the spectrum of mutations, F surpassed the other mutagens, since it induced not only changes in pathogenicity, but also small-plaque mutations.

Changes in the pathogenicity of VEE virus variants were associated

with changes in certain other genetic characteristics, which indicates the possibility of utilizing these characteristics to select and evaluate the degree of attenuation of the variants.

Five tables, 19 bibliographical entries.

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